Online Data Supplement

Expanded Methods

Animals

Mice that were wild-type (+/+, WT) or homozygous null (–/–) for targeted disruption of cyclooxygenase (COX)-2 (B6:129S7-Ptgs2^{tm1Jed}, Jackson Laboratories, Bar Harbor, ME) were studied. Littermates from heterozygous breeding were studied in hypoxia experiments. The genotypes of all COX-2 mice were determined by a PCR protocol provided by Jackson Laboratories with modification. For nimesulide experiments, C57BL/6 wild-type mice (Charles River) were studied. All animal experiments were performed in compliance with the relevant laws and guidelines as set forth by the Harvard Medical Area Standing Committee on Animals.

Chemicals and Reagents

NS-398, PGE_2, and iloprost were obtained from Cayman Chemical (Ann Arbor, MI). Platelet-derived growth factor-BB (PDGF-BB) was obtained from Peprotech Inc. (Rocky Hill, NJ). All cell culture reagents were obtained from Cellgro (Herndon, VA) unless otherwise specified. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Hypoxic Exposure and Hemodynamic Measurements

Eight- to 10-week old COX-2$^{-/-}$ and COX-2$^{+/+}$ littermates were exposed to normobaric hypoxia (10% O_2, OxyCycler chamber, Biospherix Ltd, Redfield, NY)[1,2] or normoxia (21% O_2) for 2 weeks. In separate experiments, 8-10 week old C57BL/6 WT mice were

treated with vehicle or nimesulide (40 mg/L)[3,4] in the drinking water during a 2 week exposure to hypoxia or normoxia. Following hypoxic exposure, mice were anesthetized with sodium pentobarbital (60 mg/kg) and hemodynamic measurements were performed.[2,5] The hearts were excised and the ventricles dissected and weighed. RVH was assessed by normalizing RV weight to total body weight (TBW) (RV weight/TBW).[1,2]

Histological Analysis and Immunohistochemistry

Following hemodynamic measurements, lungs were inflated, harvested, fixed in Methyl Carnoy's (10% glacial acetic acid, 60% methanol, 30% chloroform) at 4°C, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E),[6] Masson's trichrome, and immunostained for α-smooth muscle actin (α-SMA, 1:50)[7,8] and the endothelin-1 receptor (ET_AR) (1:250, BD Biosciences). The remodeled vasculature was analyzed further by injecting mice with bromodeoxyuridine (BrdU, 50 mg/kg) 16-18 h and 1-2 h prior to harvest, and staining formalin-fixed lung tissue with an anti-BrdU antibody (Dako, Carpinteria, CA) as described.[8]

Morphometry

Remodeling was quantitated as described previously[1,2,5] by capturing images of H&E-stained lung tissue (5 μm sections) with a digital camera (Leica DFC300, Allendale, NJ) coupled to a microscope and measuring arteriolar wall area with the NIH image analysis program ImageJ. Arterioles located at the level of the terminal bronchioles and adjacent to the alveolar ducts (<50 μm) were identified and at least 10 vessels were evaluated per mouse. Percent wall thickness was calculated as: wall thickness (%) = ($area_{ext}$ –

$area_{int}) \div area_{ext} \times 100$, where $area_{ext}$ represents the external diameter and $area_{int}$ represents the internal diameter of each vessel respectively.[1,2,5] PASMC hypertrophy was quantified by measuring arteriolar wall area using ImageJ ($area_{ext} – area_{int}$) and counting individual nuclei per vessel (10 vessels per mouse). PASMC hypertrophy was calculated as: vessel wall area ÷ # nuclei per vessel and reported as area per cell. ET_AR immunostaining was quantified in pulmonary arterioles using a modified version of NIH Image 1.63[9]. At least 10 vessels were identified per mouse, as described above, and the areas staining positive for ET_AR expression were measured by colorimetric analysis[9].

Western Blot Analysis

Protein extracts from lungs exposed to hypoxia or normoxia were analyzed by Western blot analysis[8,10] with a monoclonal α-SMA antibody (1:2000, Sigma), a monoclonal endothelin-1 receptor (ET_A) antibody (1:500, BD Biosciences), a polyclonal COX-2 antibody (1:200, Cayman), and a polyclonal COX-1 antibody (1:1000, Cayman). Equal loading was confirmed with an anti-tubulin antibody (1:8000, Sigma).

Cell culture

Primary aortic smooth muscle cells (VSMC) were isolated from COX-$2^{-/-}$ and COX-$2^{+/+}$ embryos at 18.5 dpc as described[8,11] and cultured in DMEM containing 20% FBS, 10,000 IU/mL penicillin, 10,000 μg/mL streptomycin, 29.2 mg/mL L-glutamine, 1 mM sodium pyruvate, and 1X MEM non-essential amino acids. Experiments were performed on VSMC at passages 4-6. Primary PASMC were isolated from adult (8-10 week old)

COX-2$^{-/-}$ and COX-2$^{+/+}$ mice as described with modification[12,13] and cultured as above. Experiments were performed on PASMC at passages 3-4. Rat pulmonary artery smooth muscle cells (RPASMC)[14] were cultured in M199 (Mediatech Inc., Herndon, VA) containing 20% FBS, 10,000 IU/mL penicillin, 10,000 μg/mL streptomycin and 29.2 mg/mL L-glutamine in a humidified incubator (21% O_2, 5% CO_2) at 37° C. Hypoxia experiments were performed in an Invivo2 400 Hypoxia Workstation (Biotrace International BioProducts, Bothell, WA) at 1% O_2, 5% CO_2, 94% N_2 at 37° C.[15]

ELISA

COX-2$^{-/-}$ and COX-2$^{+/+}$ PASMC and VSMC were exposed to hypoxia (1%) for 24 h. Supernatants were isolated and analyzed for PGE_2 and 6-keto-$PGF_{1\alpha}$ by ELISA (Elisatech, CO).

Proliferation and Migration Assays

To assess proliferation, COX-2$^{-/-}$ and COX-2$^{+/+}$ VSMC were serum starved (0.5% FBS) for 48 h, stimulated with PDGF-BB (25 ng/mL), then exposed to hypoxia (1% O_2)[15] or normoxia. Cells were counted at serial time points following hypoxia with a Beckman Coulter Counter (Beckman Coulter, Fullerton, CA).[16] To assess the ability of COX-2$^{-/-}$ and COX-2$^{+/+}$ VSMC to migrate in response to a chemoattractant, serum-starved cells were placed in the upper chamber of a 6-well transwell plate (Costar, 8-μm pore size) in triplicate (300,000 cells/well). The bottom chamber was filled with 0.5% FBS medium containing 25 ng/mL of PDGF-BB and cells were exposed to hypoxia or normoxia. Migrated cells were trypsinized and counted at serial time points with a Coulter

Counter.[8] The percentage of migrated cells was calculated as the number of cells that migrated to the underside divided by the total cell number initially plated in the upper chamber × 100.

Traction force microscopy

Contractile forces exerted by COX-2$^{-/-}$ and COX-2$^{+/+}$ VSMC were assessed by traction force microscopy as described.[17-19] Briefly, cells (1.5×10^3) were plated on polyacrylamide gel substrates containing green fluorescent nanobeads (500 nm diameter; Molecular Probes, Eugene, OR) and coated with fibronectin (5 mg/cm^2, Collaborative Research, Bedford, MA).[17-19] Cells were exposed to hypoxia (1%) or normoxia for 24 h and in certain experiments, cells were treated with ET-1 (20 nM). Traction forces exerted by VSMC were quantitated and spatially mapped by measuring bead position before and after 1% SDS, and in some experiments, before and after ET-1, calculating relative bead displacements and determining the Young's modulus of the gel, as previously described.[17-19]

Collagen matrix contraction assay

To assess contractility of COX-2$^{-/-}$ and COX-2$^{+/+}$ PASMC and VSMC on a collagen gel matrix, 2×10^5 cells were plated on type I collagen gel matrices in 24-well dishes in triplicate and exposed to hypoxia or normoxia for 24 h. Collagen matrices were prepared[20] with Vitrogen 100 collagen (Celltrix, Santa Clara, CA), 10X DMEM, and HEPES (pH 8.5). After hypoxic exposure, the matrix was released to initiate contraction and then incubated in normoxia at 37°C. Gel size was defined as the sum of the two

longest gel diameters and gel contraction expressed as a percentage of the original gel size.[20] In certain experiments, cells were treated with either PGE_2 (1 μM), iloprost (1 μM), or vehicle (30% ethanol in PBS) during hypoxic exposure. In other experiments, cells were treated with either forskolin (10 μM), an activator of adenylate cyclase, or vehicle (4% ethanol in DMEM) during hypoxic exposure. In RPASMC experiments, cells were treated with NS-398 (5 μM) or vehicle (25% DMSO in PBS).

References

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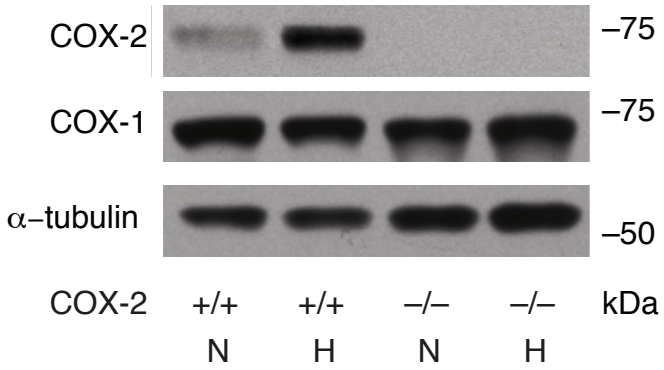
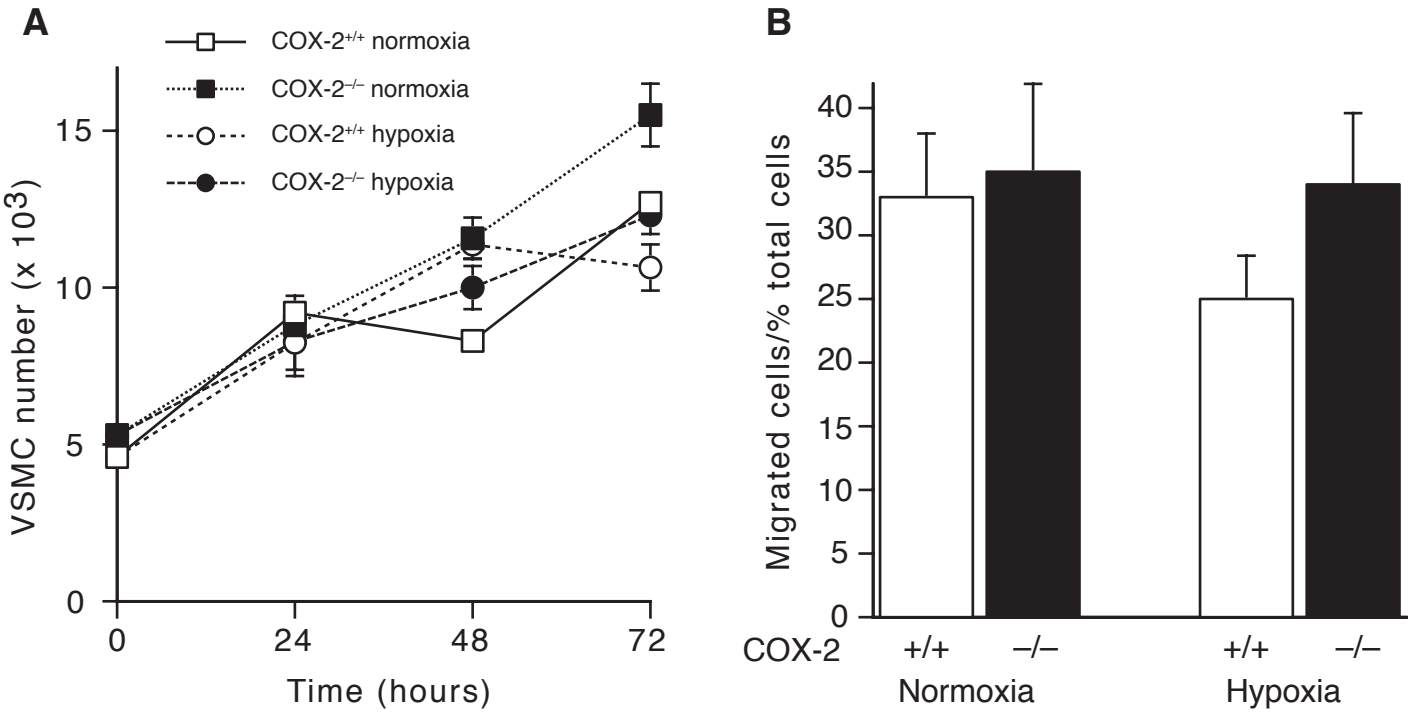
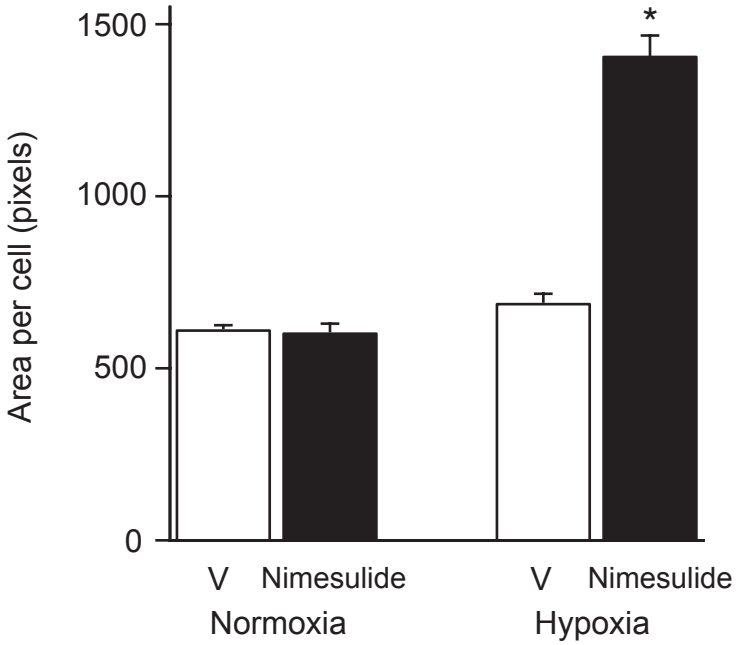


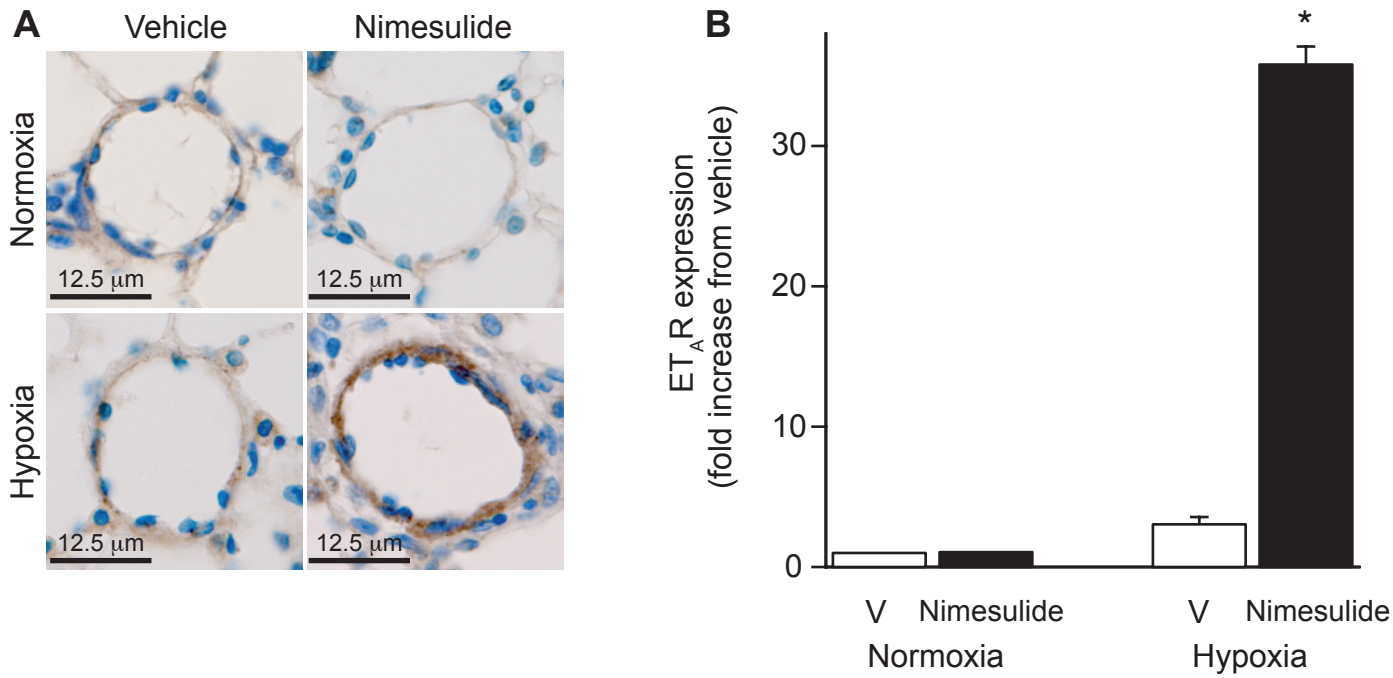
Supplemental Figure 1. Induction of COX-2, but not COX-1, in the lung following hypoxia. COX-$2^{+/+}$ and COX-$2^{-/-}$ mice were exposed to normobaric hypoxia (10% O_2) or normoxia (21% O_2) for two weeks. Lungs were harvested and total protein isolated. Western blot analysis was performed for COX-2 and COX-1. Equal loading was confirmed with an anti-α-tubulin antibody.



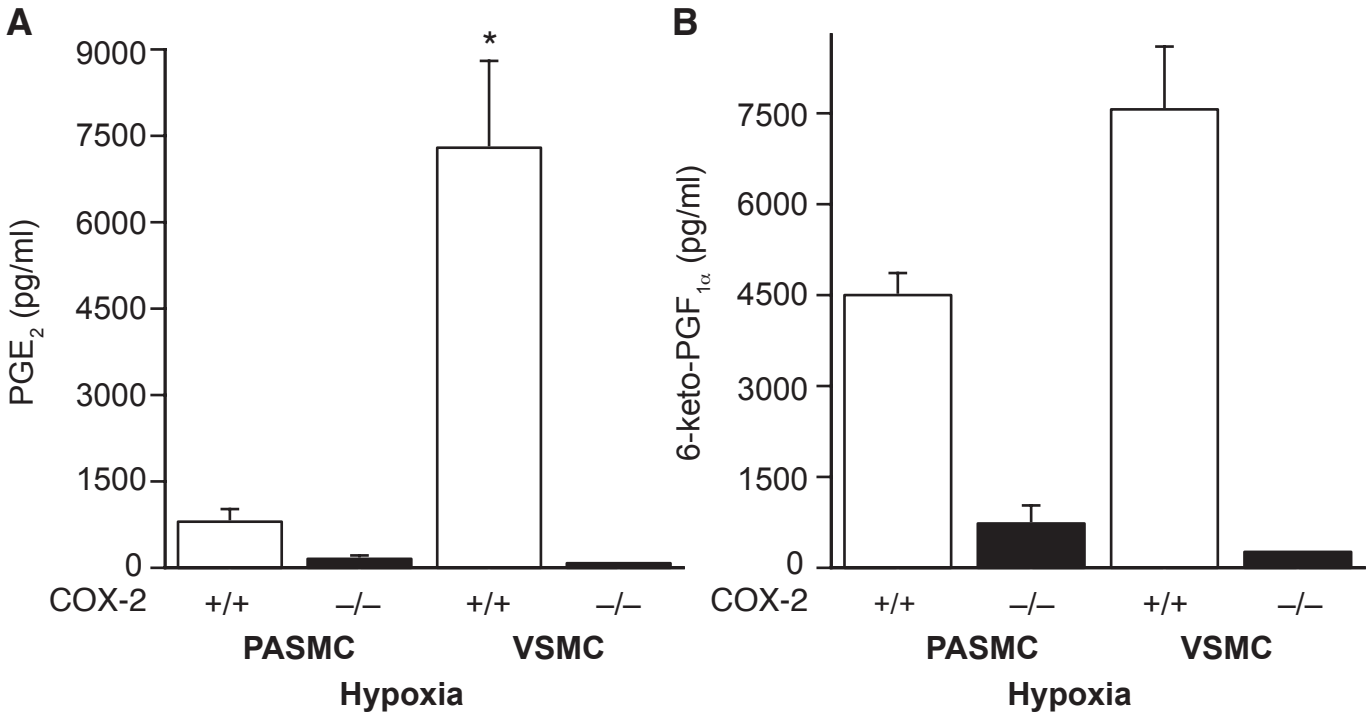
Supplemental Figure 2. Neither proliferation nor migration differ between COX-2$^{-/-}$ and COX-2$^{+/+}$ VSMC exposed to hypoxia. A) Serum-starved COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMC were stimulated with PDGF-BB (25 ng/ml) and exposed to hypoxia or normoxia. Cell number was determined at serial time points following hypoxia (COX-2$^{+/+}$ ○, COX-2$^{-/-}$ ●) and normoxia (COX-2$^{+/+}$ □, COX-2$^{-/-}$ ■). **B)** COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMC were serum starved, placed in hypoxia or normoxia, then exposed to PDGF-BB in transwell chamber plates. Migrated cells were quantitated 4 h following PDGF under hypoxic and normoxic conditions. Values are mean ± SE of 3 experiments.



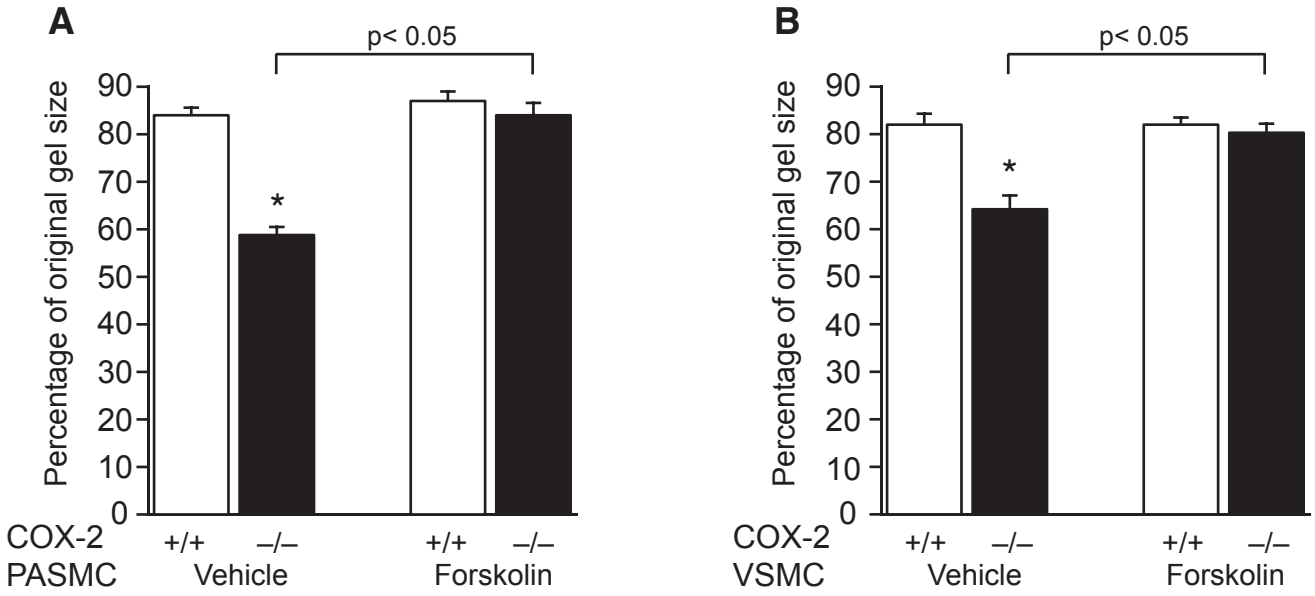
Supplemental Figure 3. Inhibition of COX-2 during hypoxia leads to enhanced PASMC hypertrophy. Quantitation of PASMC size in vehicle-treated (V) and nimesulide-treated WT mice following normoxia (n=6 per group) and hypoxia (n=10 per group). Ten vessels were analyzed per mouse. Data are expressed as mean ± SE (*$p<0.05$ for nimesulide vs. vehicle-treated hypoxic WT mice and normoxic controls).



Supplemental Figure 4. Inhibition of COX-2 during hypoxia leads to enhanced ET_A receptor expression. A) Immunostaining of lungs from vehicle-treated (left) and nimesulide-treated (right) WT mice following normoxia (top) and hypoxia (bottom) for the ET_A receptor (ET_AR). **B)** Quantitation of ET_AR expression in lungs of vehicle-treated (n=6 per group) and nimesulide-treated (n=10 per group) WT mice. Ten vessels were analyzed per mouse. Data are expressed as mean ± SE (*$p<0.05$ for nimesulide vs. vehicle-treated hypoxic WT mice and normoxic controls).



Supplemental Figure 5. Levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ in PASMC and VSMC following hypoxia. COX-$2^{+/+}$ and COX-$2^{-/-}$ PASMC and VSMC were exposed to hypoxia (1% O_2) for 24 h. Supernatants were isolated and PGE_2 and 6-keto-$PGF_{1\alpha}$ levels assayed by ELISA. Values are mean ± SE of 3 independent experiments (*$p<0.05$ for WT VSMC vs. WT PASMC).



Supplemental Figure 6. Activation of adenylate cyclase attenuates contractility of COX-2$^{-/-}$ PASMC and VSMC on collagen gels following hypoxia. Gel contraction by COX-2$^{+/+}$ and COX-2$^{-/-}$ PASMC **(A)** and VSMC **(B)** following treatment with forskolin under hypoxic conditions. Data are presented as the percentage of the original collagen gel size for hypoxic PASMC and VSMC (COX-2$^{+/+}$ □ and COX-2$^{-/-}$ ■) treated with forskolin or vehicle. Data are expressed as mean ± SE (*p<0.05 for vehicle-treated COX-2$^{-/-}$ PASMC vs. vehicle-treated COX-2$^{+/+}$ PASMC, p<0.05 for forskolin-treated COX-2$^{-/-}$ PASMC vs. vehicle-treated COX-2$^{-/-}$ PASMC **(A)**; *p<0.05 for vehicle-treated COX-2$^{-/-}$ VSMC vs. vehicle-treated COX-2$^{+/+}$ VSMC, p<0.05 for forskolin-treated COX-2$^{-/-}$ VSMC vs. vehicle-treated COX-2$^{-/-}$ VSMC **(B)**).